LABORATORY METHODS

IN THIS CHAPTER

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1. Appropriate Methods for Susceptibility Testing

There are a variety of methods with which a laboratory can determine the antimicrobial susceptibility of *S. pneumoniae*. The most common methods include disk diffusion, agar dilution, broth microdilution, and testing by antimicrobial gradient agar strips (Etest® method). Disk diffusion is a qualitative test, and if performed appropriately, the diameter of the zone of growth inhibition around an antibiotic disk will reliably predict the *in vivo* effectiveness of many antimicrobial agents. Agar dilution and broth microdilution are two quantitative methods used

for determining the minimal inhibitory concentration (MIC), and are commonly considered to be 'gold standard' reference testing methodologies. The Etest® is a semi-quantitative test that uses the principle of disk diffusion with a standard antimicrobial gradient on a plastic strip, providing an MIC usually accurate within one to two dilutions of a reference method.

Once the MIC or zone diameter of an isolate to an antimicrobial agent has been measured, the isolate is then classified to indicate whether the infection it causes may fail to respond to therapy with that agent. For clinical applications, antimicrobial susceptibilities are always interpreted strictly according to standardized guidelines (e.g. NCCLS interpretive criteria). It is important that laboratorians note the source of the pneumococcal isolate on which antimicrobial susceptibility testing is being performed, because as of 2002 there are breakpoints that differ for meningitis (CSF) isolates for some organism-antimicrobial agent combinations. (1)

Not all antimicrobial susceptibility testing methods are equally suitable for all combinations of antimicrobial agent and organism, and the disk diffusion method gives valid data for only certain antibiotics. One key example is that the oxacillin disk (1μg) can be used to predict susceptibility of a pneumococcal isolate to the penicillins and some cephlasphorins and carbopenems. It cannot be used to infer if an isolate is non-susceptible. That is, if an isolate has a inhibition zone diameter of $\geq$20mm surrounding a 1 μg oxacillin disk, it is "susceptible", but if the diameter of the zone of inhibition of growth is <20 mm, an MIC testing method is required in order to identify the isolate as susceptible, intermediate or resistant to the penicillins and cephalosporins. Therefore isolates from patients with meningitis or sepsis should not undergo preliminary antimicrobial susceptibility testing with the oxacillin disk because of the need for definitive susceptibility results for rapid clinical decision-making.

2. NCCLS Definitions for *susceptible, intermediate, resistant*

An organism that is classified as "resistant" to an antimicrobial agent can cause an infection that fails to respond to therapy with the recommended dose of the antimicrobial agent ("treatment failure"). Laboratories should report the measurement of susceptibility (in mm for disk diffusion methods and in μg/ml for MIC methodologies) along with the corresponding interpretations as sensitive, intermediate, or resistant ("S/I/R"). Because treatment decisions may vary on clinical syndrome and severity of illness (2,3) inclusion of the actual susceptibility measurement can assist clinicians with therapeutic decisions.

3. Which Drugs to Test and Monitor

Selection of the most appropriate antimicrobial agents to test and monitor is a decision best made by each clinical laboratory in consultation with infectious disease practitioners and the pharmacy, as well as the pharmacy and therapeutics and infection control committees of the medical staff. (4) The NCCLS recommendations found in Table I (please see Attachments at end of Laboratory Methods section) for *S. pneumoniae* comprise agents of proven efficacy that show acceptable *in vitro* test performance. Considerations for specific drugs to comprise a panel for testing and reporting should include: clinical efficacy, prevalence of resistance, minimizing emergence of resistance, cost, FDA indications, and current consensus recommendations for first choice and alternative drugs, in addition to other specific issues (5). The listing of antimicrobial agents represent recommendations for testing and reporting that are considered appropriate at the present time. To reduce potential for misinterpretation of results, routine reports should only include those antimicrobials appropriate for treatment. Agents may be added or removed as conditions warrant.

4. Reporting Susceptibility Testing Results

CDC recommends that laboratory reports include the following information, important for both clinical and quality assurance purposes.

- *Method used to perform antimicrobial susceptibility testing* (*i.e.*, agar dilution, broth microdilution, disk diffusion, Etest®).
- *Separate data fields for the MIC* (measured in μg/ml) or the diameter of the zone of inhibition of growth (measured in mm) *and the corresponding interpretation* of that value as susceptible, intermediate, or resistant (S/I/R).
- *Separate fields for the results of susceptibility testing of each antimicrobial agent* obtained by the laboratory and also the results for the same isolate-antimicrobial agent combinations if re-tested by a reference laboratory.

The laboratory database should include the results of all antimicrobial agents tested, including those agents that may not be routinely reported to clinicians when selective suppression or cascade reporting algorithms are applied.

5. Quality Assurance

Quality control tests should be performed as a part of the normal laboratory routine. To verify that susceptibility tests results are accurate, it is important to include at least one control organism with each test or new set of testing conditions. ATCC 49619 is the NCCLS control strain to use when testing *S. pneumoniae*. Zone diameters obtained for the control strain should be compared with NCCLS published limits. For *S. pneumoniae*, the CDC also recommends

using a reference control strain (i.e., ATCC® 49619) to show resistance. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

Quality control tests should be preformed once per week if susceptibility tests are performed daily, or with every group of tests when testing is done less frequently. They should also be done with each new batch of susceptibility test medium and every time a new lot of disks is used.

The accuracy and reproducibility of antimicrobial susceptibility tests are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis. All techniques require strict adherence to a quality-controlled protocol for results to be meaningful.

Medium requirements

The proper test medium for antimicrobial susceptibility testing of *S. pneumoniae* by the disk diffusion or Etest® methods is Mueller-Hinton agar plus 5% sheep blood (although horse blood should be used when testing susceptibility to trimethoprim-sulfamethoxazole). The proper test medium for antimicrobial susceptibility testing of *S. pneumoniae* using MIC dilution methods is cation-adjusted Muller-Hinton broth plus 2% to 5% (vol/vol) lysed horse blood. These test media must be used in order for NCCLS methods and interpretive criteria to be applied. (See Table II for antimicrobial susceptibility test breakpoints and quality control ranges for *S. pneumoniae*.)

6. Pros and Cons of Isolate Collection

Whether or not to collect isolates from clinical laboratories to retest susceptibility at the state health department laboratory or another reference laboratory is a decision that state health department personnel must make based on specific surveillance goals. Factors that weigh into the decision to collect pneumococcal isolates include the following:

- Are clinical laboratories using appropriate methods for testing pneumococci for resistance?
- How do susceptibility results from clinical laboratories compare with results from reference labs?
- What drugs are clinical laboratories testing?

The negative aspects of collecting pneumococcal isolates include the work and cost involved in collecting, testing, and storing isolates. Surveillance personnel must coordinate transport and logistical requirements with participating collection sites. This requires a significant investment in time as well as money. In order to test isolates, a minimum standard of laboratory capacity and laboratory personnel training must be provided, both requiring a substantial financial investment.

However, if state health department personnel conclude that their capacity allows for isolate collection, there are various public health benefits derived from the additional workload. Isolate collection does increase the richness of available data for analysis. It also allows for use of standard testing methods at the state or reference laboratory level. Laboratory personnel may also test a variety of drugs, and perform specialized tests in the state laboratory.

In 2000, CDC conducted a survey of clinical laboratories susceptibility testing practices in ABCs sites (CDC, unpublished data). The objectives were to assess whether clinical laboratories used NCCLS-recommended testing methods for susceptibility testing of sterile-site pneumococci and to determine which drugs were tested. The survey was sent to all clinical laboratories (n=659) in nine ABCs areas in 2000. Questions addressed methods, drugs, tested and reporting practices. Of the 547 laboratories (83%) that responded to the survey, 357 (78%) did some pneumococcal susceptibility testing in-house. Half reported starting with an oxacillin screen, even for isolates from blood and CSF. The antibiotics most frequently included in susceptibility testing were penicillin, cefotaxime/ceftriaxone, and vancomycin. Few (40%) of the laboratories were routinely performing testing for susceptibility to flouroquinolones.

A second study compared MIC results for *S. pneumoniae* from 877 clinical and reference laboratories, expressed in terms of dilution differences (CDC, unpublished data). Preliminary results for comparisons of seven antibiotics showed generally good agreement between local and reference results. In general, clinical laboratory results were within 1 dilution of reference laboratory results, the allowable margin of error for the test. Errors of >1 dilution from reference results were most common with cefotaxime (5%), erythromycin (15%), clindamycin (21%), and TMP/sulfa (11%). Very major errors, errors in which a reference laboratory called an isolate resistant and the clinical laboratory called it susceptible, were most common with erythromycin (3%), and cotrimoxazole (2%).

The assumption from these studies is that clinical laboratory pneumococcal susceptibility results are generally reliable. The problem is that relying on clinical laboratory results means limited

information for some drugs of interest (e.g., fluoroquinolones). The decision on whether or not to collect isolates requires weighing the benefits against costs and workload.

Performing susceptibility testing on isolates may be a useful adjunct to surveillance as part of a vaccine program. The additional work volume resulting from susceptibility testing could be limited to evaluation of cases in children <5 years, the target population for the conjugate vaccine, for evaluation of pneumococcal outbreaks.

7. Laboratory Support for Surveillance of Vaccine Preventable Pneumococcal Infections

Some cases of invasive pneumococcal disease following pneumococcal conjugate (PCV7) vaccination are to be expected, since vaccine efficacy was 97% for invasive disease with pneumococcal serotypes included in the vaccine and 89% for all serotypes. The Respiratory Diseases Branch (RDB) of CDC has developed a tracking system to determine the serotype of invasive pneumococcal isolates, record host conditions that may contribute to PCV7 failure, and to monitor for vaccine lots that may be associated with decreased protection. The tracking system is consistent with the 2000 CSTE position statement on invasive pneumococcal infections, which recommends that state health departments monitor invasive pneumococcal disease in children less than 5 years old.

The Pneumococcal Conjugate Vaccine Failure Case Report Form may be submitted when the following five conditions are met:

- The child is <5 years old
- The child has an invasive pneumococcal infection, defined as isolation of *S. pneumoniae* from a normally sterile site (e.g., CSF, blood, joint fluid, pericardial fluid)

- A pneumococcal isolate is available for serotyping,
- A PCV7 vaccine history is available, and
- The child has received at least one dose of PCV7

If all five conditions are met, a completed PCV7 failure case report form, lab form and isolate should be sent to the CDC Streptococcus laboratory through your state health department. The CDC disease reporting instruction sheet and a case report form are available on line at http://www.cdc.gov/nip/diseases/pneumo/PCV-survrpts/PCV7-instructions.htm. Cases of suspected PCV7 failure may also be reported to the Vaccine Adverse Events Reporting System (VAERS) at http://www.vaers.org. Reporting through VAERS is not required. However if a clinically significant adverse event occurs after vaccination with PCV7, it should be reported. Isolates will be serotyped and results of the serotyping will be returned to the state health department and submitting physician or laboratorian.

Data on serotypes can be useful for surveillance of vaccine failures. Since most state laboratories do not currently perform serotyping, in cases of vaccine failure, state laboratory personnel may submit isolates to CDC for serotyping. States that are interested in developing serotyping programs may also contact CDC for assistance with serotyping training.

8. When to Contact CDC Streptococcus Laboratory for Isolate Retesting

State health department personnel are encouraged to contact CDC's Streptococcus Laboratory for assistance or serotyping requests for cultures meeting the following requirements:

1. All pneumococcal isolates from patients having received one or more doses of the pneumococcal conjugate vaccine. Isolates should be sent with the appropriate reporting form found at http://www.cdc.gov/nip/diseases/pneumo/PCV-survrpts/PCV7-form.pdf
2. Any culture that fails to type when using the complete set of typing antisera. Since an appreciable number of strains will not type when using the checkerboard method, CDC should be contacted for serotyping assistance only after testing with the complete set of typing antisera has been performed.
3. All cultures with unusual antimicrobial resistance patterns, such as an isolate with a vancomycin MIC greater than or equal to 2.0 mg/ml or penicillin MIC with a breakpoint greater than or equal to 16.0 mg/ml, should contact Dr. Richard Facklam, CDC Streptococcus Laboratory, at (404) 639-0856 or the Respiratory Diseases Branch at (404) 639-2215.

Table I: Suggested Groupings of U.S. FDA-Approved Antimicrobial Agents That Should be Considered for Routine Testing and Reporting of *S. pneumoniae* (7)

	AGENT	COMMENTS
GROUP A PRIMARY TEST AND REPORT	Erythromycin	Susceptibility and resistance to azithromycin, clarithromycin, and dirithromycin can be predicted by testing erythromycin.
	Penicillin	Only results of testing with penicillin, cefotaxime, ceftriaxone, meropenem, and vancomycin should be reported routinely for CSF isolates of *S. pneumoniae*
	Trimethoprim-sulfamethoxazole	
GROUP B[1] PRIMARY TEST REPORT SELECTIVELY	Cefepime Cefotaxime or Ceftriaxone	Only results of testing with penicillin, cefotaxime, ceftriaxone, meropenem, and vancomycin should be reported routinely for CSF isolates of *S. pneumoniae*
	Clindamycin	
	Gatifloxacin or levofloxacin or moxifloacin or sparfloxacin Ofloxacin	
	Meropenem	Only results of testing with penicillin, cefotaxime, ceftriaxone, meropenem, and vancomycin should be reported routinely for CSF isolates of *S. pneumoniae*
	Tetracycline	Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline
	Vancomycin	Only results of testing with penicillin, cefotaxime, ceftriaxone, meropenem, and vancomycin should be reported routinely for CSF isolates of *S. pneumoniae*
GROUP C [2] SUPPLEMENTAL REPORT SELECTIVELY	Amoxicillin or Amoxicillin-clavulanic acid	
	Cefuroxime	
	Chloramphenicol	
	Ertapenem Imipenem	
	Linezolid	
	Rifampin	***Rx:*** Rifampin should not be used alone for chemotherapy

Note 1: Selection of the most appropriate antimicrobial agents to test and to report is a decision made best by each clinical laboratory in consultation with infectious disease practitioners and the pharmacy, as

well as the pharmacy and therapeutics and infection control committees of the medical staff. The lists for each organism group comprise agents of proven efficacy that show acceptable in vitro test performance. Considerations in the assignment of agents to Group A, B, and C include clinical efficacy, prevalence of resistance, minimizing emergence of resistance, costs, and current consensus recommendations for first-choice and alternative drugs, in addition to the specific comments in footnotes "1" and "2." Tests on selected agents may be useful for infection control purposes.

Note 2: The boxes in the table designate clusters of comparable agents that need not be duplicated in testing, because interpretive results are usually similar and clinical efficacy comparable. In addition, an "or" designates a related group of agents that has an almost identical spectrum of activity and interpretive results, and for which cross-resistance and susceptibility are nearly complete. Therefore, usually only one of the agents within each selected box (cluster or related group) need be selected for testing. Agents that are reported must be tested, unless reporting based on testing another agent provides a more accurate result, and usually, they should match those included in the hospital formulary; or else the report should include footnotes indicating the agents that usually show comparable results. Lastly, unexpected results should be considered for reporting.

Note 3: Information in boldface type is considered tentative for one year.

Footnotes

[1] Group B represents agents that may warrant primary testing but which should be reported only selectively, such as when the organism is resistant to agents of the same class in Group A. Other indications for reporting the result might include selected specimen sources (e.g., third-generation cephalosporin for isolates of *H. influenzae* from CSF); stated allergy or intolerance, or failure to respond to an agent in Group A; polymicrobial infections; infections involving multiple sites with different microorganisms; or reports to infection control for epidemiologic aid.

[2] Group C represents alternative or supplemental antimicrobial agents that may require testing in those institutions that harbor endemic or epidemic strains resistant to one or more of the primary drugs (especially in the same class, e.g., β-lactams), or for treatment of unusual organisms, or reporting to infection control as an epidemiologic aid.

Table II: Antimicrobial susceptibility test breakpoints and quality control ranges for *S. pneumoniae* according to 2003 NCCLS standards (8)

Antimicrobial agent	*Breakpoints for Zone of Inhibition (mm) and Equivalent MIC (μg/ml)* [a]			*NCCLS QC strain ATCC 49619*
	Susceptible	**Intermediate**	**Resistant**	
Chloramphenicol (30 μg disk)	≥ 21 mm ≤ 4 μg/ml	~ ~	≤ 20 mm ≥ 8 μg/ml	23 – 27 mm 2 – 8 μg/ml
Erythromycin	≤ 15	18-20	≥ 21	
Vancomycin	-	-	≥ 17	
Tetracycline	≤ 18	19-22	≥ 23	
Levofloxacin	≤ 13	14-16	≥ 17	
Trimethoprim-Sulfamethoxazole (1.25/23.75 μg disk)	≥ 19 mm ≤ 0.5 – 9.5 μg/ml	16 – 18 mm 1/19 – 2/38 μg/ml	≤ 15 mm ≥4/76 μg/ml	20 – 28 mm 0.12/2.4 –1/19 μg/ml
Oxacillin [c] (1 μg disk) *Disk diffusion ONLY*	≥ 20 mm [c]	** [c]	** [c]	≤ 12mm [d]
Penicillin [e] *MIC testing ONLY*	≤ 0.06 μg/ml	0.12 – 1 μg/ml	≥ 2 μg/ml	0.25 μg/ml – 1 μg/ml
Ceftriaxone [f] ***Non-meningitis isolate MIC***	*<disk not tested>* ≤ 1 μg/ml	*<disk not tested>* 2 μg/ml	*<disk not tested>* ≥ 4 μg/ml	30 – 35 mm 0.03 – 0.12 μg/ml
Meningitis isolate MIC	≤ 0.5 μg/ml	1 μg/ml	≥ 2 μg/ml	
Cefotaxime [f] ***Non-meningitis isolate MIC***	*<disk not tested>* ≤ 1 μg/ml	*<disk not tested>* 2 μg/ml	*<disk not tested>* ≥ 4 μg/ml	31 – 39 mm 0.03 – 0.12 μg/ml
Meningitis isolate MIC	≤ 0.5 μg/ml	1 μg/ml	≥ 2 μg/ml	

* Source: NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement*. NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

*** Oxacillin (1μg) disks are used to predict susceptibility to the β-lactam drugs; the MIC presented is based on penicillin. For Oxacillin, when the zone of inhibition is smaller than 20 mm, further testing is called for to get an MIC. Some of these strains will actually be susceptible.*

^ According to the NCCLS 2002 M100-S12 publication: "Deterioration in Oxacillin disk content is best assessed with

QC organism Staphylococcus aureus ATCC 25923, with an acceptable zone diameter of 18 – 24 mm.

References

1. NCCLS (2003) *Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

2. Gonzales R, Bartlett JG, Besser RE, et al. Principles of appropriate antibiotic use for treatment of acute respiratory tract infections in adults: background, specific aims, and methods. *Ann Intern Med*. 2001; 134:479-486.

3. The National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Susceptibility Testing. Eleventh Informational Supplement. NCCLS Document M100-S11. 940 West Valley Road, suite 1400, Wayne, PA 19087, 2000.

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6. CDC. Assessment of susceptibility testing practices for *Streptococcus pneumoniae*-United States, February 2000. *MMWR*. 2002.51(18);392-4.

7. CDC. Susceptibility Testing Practices for *Streptococcus pneumoniae*: Report of a Survey of Laboratories Participating in Active Bacterial Core Surveillance (ABCs), February 2000

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